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Determination of antibiotics from soil by pressurized liquid extraction and liquid chromatography–tandem mass spectrometry

Michael P. Schlüsener, Michael Spiteller, Kai Bester*

Institute of Environmental Research (INFU), University of Dortmund, Otto-Hahn Strasse 6, D-44227 Dortmund, Germany

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Abstract

A method for the analysis of several macrolide and ionophore antibiotics, as well as tiamulin, from soil was developed using pressurized liquid extraction (PLE), reversed-phase liquid chromatography and atmospheric pressure chemical ionisation (APCI) tandem mass spectrometry (LC–APCI$^+$-MS–MS). The analytes were extracted from soil by PLE in 30 min and the extracts were cleaned up by solid-phase extraction (SPE) on a diol SPE cartridge. Liquid chromatographic (LC) separation of the antibiotics was achieved in 35 min. Recovery experiments were performed using spiked soil and concentrations varying from 1 to 2000 μg/kg. By using a macrolide internal standard the recovery rates for the macrolides erythromycin and roxithromycin ranged from 43 to 94% (RSD 20–23%), for the ionophore salinomycin the recovery rate was 76% (RSD 29%), while the pleuromutilin tiamulin was completely recovered. The limits of detection ranged from 0.2 to 1.6 μg/kg. In soil samples a maximum concentration of 0.7 μg/kg tiamulin was found.

Keywords: Pressurized liquid extraction; Antibiotics; Macrolides

1. Introduction

More than 5100 t of antibiotic agents were used in large-scale animal husbandry and 5400 t in human medicine in Europe during 1997 [1]. As antibacterial substances these compounds were designed to cause a biological effect, thus they may exhibit adverse effects on water and soil organisms when reaching the environment. Several antibiotics have been detected in the environment [2–4] and toxic effects on fauna have also been observed [5,6]. Resistance of microorganisms against the majority of existing antibiotics was reported by Neu [7].

Antibiotics have been widely used in human and veterinary medicine to prevent or to treat microbial infections as well as in livestock production to promote the growth of animals. Growth promotion with sodium-monensin, sodium-salinomycin, flavophospholipol and avilamycin will be phased out in the EU on 1 January 2006 [8]. After administration, 50–90% of these pharmaceuticals or their primary metabolites are excreted rapidly by humans or animals [9]. Thus, large quantities of these antibiotics are transferred after application and excretion together with liquid manure to holding tanks. They are introduced into the environment when manure is sprayed on fields. Little is known about the occurrence and fate of antibiotics in soil. These pharmaceuticals may accumulate in soil [10,11] and

*Corresponding author. Fax: +49-231-755-4084.
E-mail address: k.bester@infu.uni-dortmund.de (K. Bester).

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influence soil organisms [6]. On the other hand, a very hydrophilic drug may be mobile in soil and can contaminate ground water.

A reliable method to determine the more recently used antibiotics with macrocyclic, polyether or pleuromutilin structures has not yet been described in the available literature. Some tetracyclines have been analysed by Hamscher et al. [12,13].

The aim of the present work was to develop a reproducible and sensitive multimethod to determine the commonly used macrolides erythromycin, roxithromycin, oleandomycin, tylosin and ivermectin, the ionophores salinomycin and monensin, as well as the pleuromutilin derivate tiamulin as a prerequisite for an investigation of the fate of these antibiotics in soil. The structural formulae of some of the analytes and the internal standard are shown in Fig. 1.

Several methods for the analysis of macrolides, ionophores and tiamulin in animal tissues, milk, manure and plasma have been described using liquid chromatography–ultra-violet detection (LC–UV) [14–16], liquid chromatography–mass spectrometry (LC–MS) [17–19] and liquid chromatography–tandem mass spectrometry (LC–MS–MS) [20,21].

As soil is a complex matrix, exhaustive extraction is important to obtain high recovery rates and an efficient clean-up procedure is necessary to remove interfering matrix components.

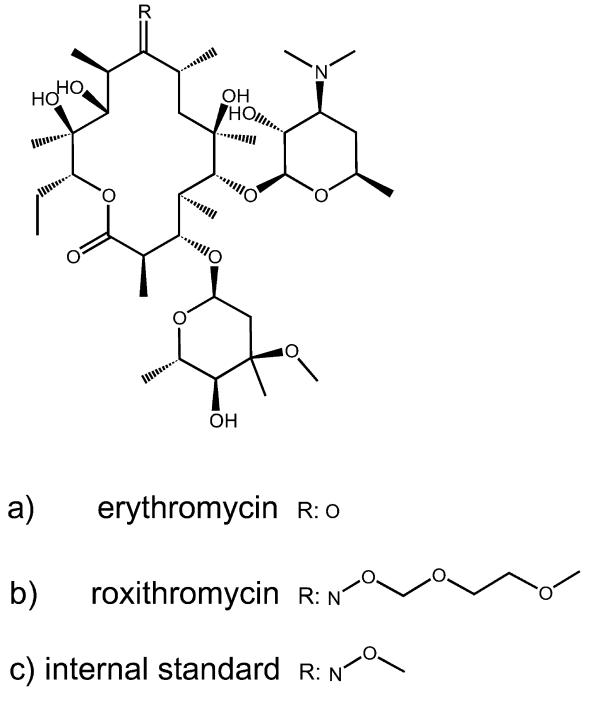

Fig. 1. Structural formulae of the macrolides, (a) erythromycin, (b) roxithromycin, and (c) internal standard.

2. Experimental

2.1. Materials

Acetonitrile (HPLC-S gradient grade) was purchased from Biosolv (Valkenswaard, Netherlands). Water (HPLC grade) was obtained from Mallinckrodt Baker (Griesheim, Germany). Methanol (suprasolv grade) and acetone (analytical grade) were obtained from Merck (Darmstadt, Germany).

Ammonium acetate, aqueous ammonia solution (25%) and glacial acetic acid were of analytical grade and were purchased from Merck. Erythromycin, ivermectin, roxithromycin and tylosin tartrate were provided by Sigma–Aldrich (Seelze, Germany). Oleandomycin phosphate dihydrate, monensin sodium salt, salinomycin SV sodium salt 2.5-hydrate and tiamulin furmarate (Vetranal™) were obtained from Riedel-de Haën (Seelze, Germany).

2.2. Internal standard

The synthesis of (*E*)-9-[*O*-(2-methyloxime)]-erythromycin is described in Ref. [21].

2.3. LC

Separations were performed using a Phenosphere-Next RP18 column (150×2 mm I.D., particle size 3 μm) and a SecurityGuard (Phenomenex, Torrance, CA, USA) at 25±1 °C. The flow rate was 0.2 ml/min. The LC gradient was established by mixing two mobile phases: phase A, 0.1 *M* aqueous ammonium acetate solution and phase B, pure acetonitrile. Chromatographic separation was achieved with the following gradient: 0–1 min 10% B, 1–14 min 10–100% B, 14–29 min 100% B, 29–30 min 100–10% B, 30–35 min 10% B. A 10-μl aliquot of each sample was injected. The gradient was optimised for

LC separation, but not for ionisation as it is used to suppress sodium adducts of the analytes as well.

The LC system consisted of a GINA 50 autosampler, a P 580A HPG LC pump, a DEGASYS DG-1210 degasser unit and a STG 585 column oven (all from Dionex, Idstein, Germany). The dead time of the LC system was 1.8 min. After LC separation, the analytes were determined by atmospheric pressure chemical ionisation–tandem mass spectrometry ($APCI^+$-MS–MS) in positive ion mode and selected reaction monitoring (SRM). The total flow of the LC system was directed to the APCI source.

2.4. Mass spectrometry

The triple quadrupole mass spectrometer (TSQ 7000, Finnigan-MAT, Bremen, Germany) was equipped with an APCI 2 source and operated under the following conditions: capillary temperature, 180 °C; sheath gas, 40 p.s.i.; corona current, 5 μA; vaporizer temperature, 450 °C; auxiliary gas, off; q_0 offset, −4.4 V; collision cell pressure, 2.0 mT; collision gas, argon; multiplier, 1900 V. The potential difference between the capillary and the tube lens was held at 70 V. The cycle time was 1.2 s during the chromatographic determination of antibiotics. The data obtained were processed using Xcalibur™ 1.2 software. The fused-silica capillary of the APCI 2 source was replaced by a steel capillary in order to reduce tailing of antibiotics adsorbing on the silica surface [22]. APCI was preferred because this ionisation is less vulnerable to matrix effects than ESI [23].

A post-column Valco divert valve was used to direct most of the fast eluting matrix (such as residues of salts, and hydrophilic compounds) of a sample to waste. Diverting the flow for the first 8 and the last 7 min to waste minimized contamination of the MS source. A substitute flow of 50 μl/min water acetonitrile (3:7, v/v) pumped by a LC-10 AT (Shimadzu, Duisburg, Germany) compensated for the missing flow from the LC during waste positing operation. Automatic data acquisition was triggered using a short contact closure signal of the autosampler.

To gain higher selectivity, selected reaction monitoring (SRM) was chosen. The optimal collision energy was determined by means of a software procedure controlling the automatic switching between the different voltages with a step size of 1 eV/scan and a range from −5 to −70 eV. A pre-scan voltage settings time of 2 ms and 1.0 s for one complete cycle (nine transitions) was used for SRM.

2.5. Pressurized liquid extraction (PLE)

An ASE 200 instrument (Dionex, Idstein, Germany) was used for the extractions performed as pressurised liquid extraction (PLE), also called accelerated solvent extraction (ASE). The effect of solvent type and PLE operational conditions on the recovery rates of antibiotic recovery was evaluated using spiked German soil (Monheim, Laacher Hof, AXXa, provided by Bayer Crop Protection, Leverkusen, Germany).

Soil samples (9.1% humidity) were stored at 4 °C. A 30-g sample of sieved humid soil (2 mm) was transferred into a 33-ml PLE cell. The cell was then filled with Ottawa sand (20–30 mesh, Fischer Scientific, Schwerte, Germany) to reduce the void volume. The cells were sealed at both ends with circular cellulose filters (Ref. no.: 321432, Schleicher & Schüll, Dassel, Germany) and end caps were fitted.

The prepared cells were extracted with 1% (v/v) aqueous ammonia in methanol with the following PLE conditions: preheat, 0 min; static, 10 min; flush, 70%; purge, 180 s; cycles, 2; pressure, 140 bar; temperature, 80 °C. After the extraction 25 μl of internal standard solution (I.S.) (10 mg (*E*)-9-[*O*-(2-methyloxime)]-erythromycin in 100 ml acetonitrile), followed by 400 μl of glacial acetic acid, were added to the extract and the flask was shaken by hand for 10 s. The mixture was poured in a 100-ml distilling flask and the sample volume was reduced to 5 ml using a rotary evaporator at 60 °C and 29 kPa. The residue was dissolved in 15 ml water and the volume was reduced again to 10 ml at 60 °C and 15 kPa.

2.6. SPE clean-up

Samples were cleaned up by a modification of the method developed by Delépine et al. [18]. Diol SPE cartridges from UCT (2000 mg, Bristol, PA, USA) were conditioned with 10 ml methanol followed by 10 ml water. A solid-phase extraction manifold (IST,

Grenzach-Wyhlen, Germany) with PTFE stopcock and outlet was used. The soil extract (10 ml) was passed through the cartridge at a speed of 5 ml/min (vacuum). The cartridge was washed with 10 ml water to remove interfering matrix components, e.g. salts and co-eluting matrix components. The analytes were eluted twice from the cartridge with 4 ml of an acetonitrile:0.1 *M* aqueous ammonium acetate (3:2, v/v) mixture. An aliquot of 0.8 ml of the eluate was transferred to a 1.5-ml autosampler vial for LC–MS–MS analysis.

2.7. Calibration and validation

The calibration was performed as an internal standard calibration in the presence of soil matrix to overcome matrix effects [23,24]. A typical German soil (Monheim, Laacher Hof, AXXa), which fulfils the requirements for standard pesticide registration studies, taken from the surface layer (0–10 cm) in areas with vegetation, was chosen for the recovery experiments. It had not received any antibiotic applications for at least 7 years. The cleaned-up extract of this soil was used for preparation of the standards in the presence of soil matrix for LC–MS–MS determination.

A stock solution was produced by dissolving 10 mg of the macrolides, ionophores and tiamulin in 100 ml acetonitrile. This standard solution was stored at 4 °C in the dark and was stable at least for 3 months. Calibration standards (5, 10, 50, 100, 500, 1000 and 5000 ng/ml) were made by serial dilution of the stock solution. The I.S. was added to the calibration standards in a concentration of 5 μl/ml. The respective calibration standard solution (0.5 ml) was filled in 1.5-ml LC vials and 0.5 ml soil matrix was added. The soil matrix solution was produced by extracting AXXa soil by PLE with successive SPE clean-up as described above. The calibration curves were calculated using a weighted ($1/X$) linear regression model.

2.8. Recovery experiments

For validation of the method, 100 g of antibiotic-free soil were spiked with the stock solution (1, 6, 20, 200 and 2000 μg/kg soil) using the following protocol. To avoid potential effects of solvents upon the sorption of compounds to the soil, the volumes of the application solution (1–1800 μl) were deposited evenly onto portions of ~10 g air-dry soil in porcelain dishes. The thus treated samples of soils were thoroughly mixed with a spatula until the solvent was completely evaporated (~10 min) and the respective compounds were evenly distributed. The respective 10-g samples were subsequently added to the total soil mass of the corresponding soil (100 g). These 110-g soil samples were homogenised by means of a tumbling mixer for 1 h. They were extracted within 1 h after homogenisation. Control experiments after 24 h aging of the spiked soil gave the same recovery rates with this method.

Recovery experiments for the macrolides, ionophores and tiamulin were carried out at five concentration levels in triplicate.

3. Results and discussion

All analytes were completely separated by LC. The calibration graphs show linearity in the range from the limit of quantitation (LOQ) up to 5000 ng/ml with correlation coefficients (r^2) better than 0.992.

3.1. Results of temperature and solvent optimisation of the PLE

For the optimisation of the PLE conditions, samples of 10 g soil were each spiked with 50 μl stock solution (10 mg of macrolides, ionophores and tiamulin in 100 ml acetonitrile) and placed in an 11-ml PLE cell. These samples were extracted three times into separate vials in four different experiments using acetone, acetonitrile, methanol and 1% (v/v) aqueous ammonia in methanol at temperatures varied from 40 to 120 °C in 10 °C steps (Fig. 3). These extracts were analysed directly after centrifugation (C-1200, NeoLab, Heidelberg, Germany) by LC–MS–MS. During this PLE optimisation experiment, all three extracts were analysed separately. Significant amounts of analytes were found in the second extract but no analytes were found in the third. In Fig. 3 the results of the first and second extraction of roxithromycin as an example are summarised. The

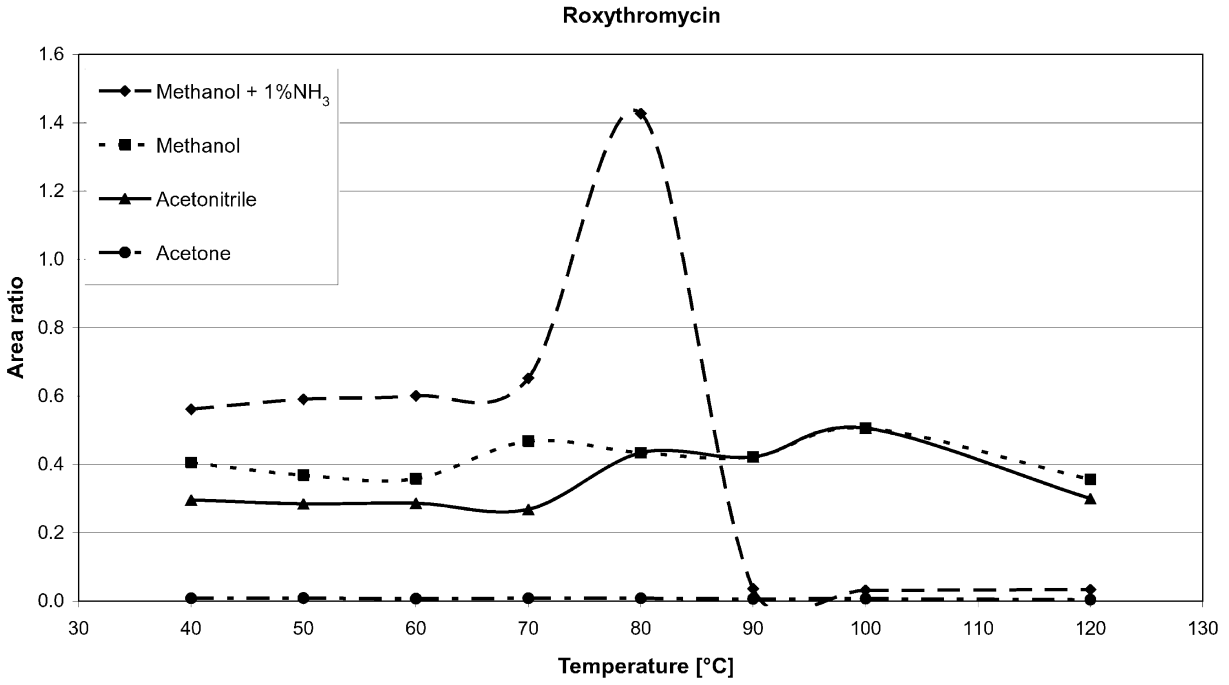

Fig. 3. Extraction of roxithromycin in spiked soil samples with different solvents at various temperatures. The graph shows the relative extraction performance ($area\ ratio = area_{\ roxithromycin} / area_{\ internal\ standard}$) depending on the temperature and the solvent. This optimisation of the ASE conditions shows the best extraction performance with methanol: 1% aqueous ammonia (v/v) at 80 °C and 140 bar.

other analytes behave similarly. For all compounds, optimum extraction efficiency was found at 80 °C and 140 bar. Above 80 °C the analytes may be hydrolysed. Temperature and pH value may help to dissolve the analytes from humic and other soil matrix compounds. Increasing the static extraction time from 10 min up to 20 min gave no higher recovery rates. To get a more homogeneous soil sample 33-ml extraction cells with 30 g sample were chosen for validation and the recovery study.

3.2. Results of recovery study

The recoveries are shown in Fig. 2. Recoveries of all experiments were averaged (Table 1) since there was no concentration (1, 6, 20, 200 and 2000 μg/kg) dependency of recoveries. For the macrolides mean recoveries of 34% (RSD 56%) to 93% (RSD 20%) were obtained. The recovery rate for salinomycin was 74% (RSD 29%), while for tiamulin it was 118% (RSD 19%). The limit of detection (LOD) was taken as a signal-to-noise ratio (S/N) of 3:1 and the limit of quantitation (LOQ) was defined as a signal-to-noise ratio of 10:1 (Table 2). This method was also tested with ivermectin, monensin and tylosin but constant recovery rates for these three antibiotics were not achieved. No blank problems were detected during the method validation or the applications.

3.3. Comparison to field data

The validated method was tested for several samples of different fields, in order to investigate the persistence of antibiotics in manure fertilised soil samples. The fields were fertilised in February 2001 with liquid manure, which contained tiamulin (43 μg/kg) and salinomycin (11 μg/kg) [21]. Additionally the amount of applied manure was given (20 m^3/ha). The samples were taken in November 2001. Fig. 4 shows the SRM trace of a soil sample which contained tiamulin. No salinomycin was detected in these soil samples, though salinomycin was applied at the farm.

The concentration of tiamulin was in the same

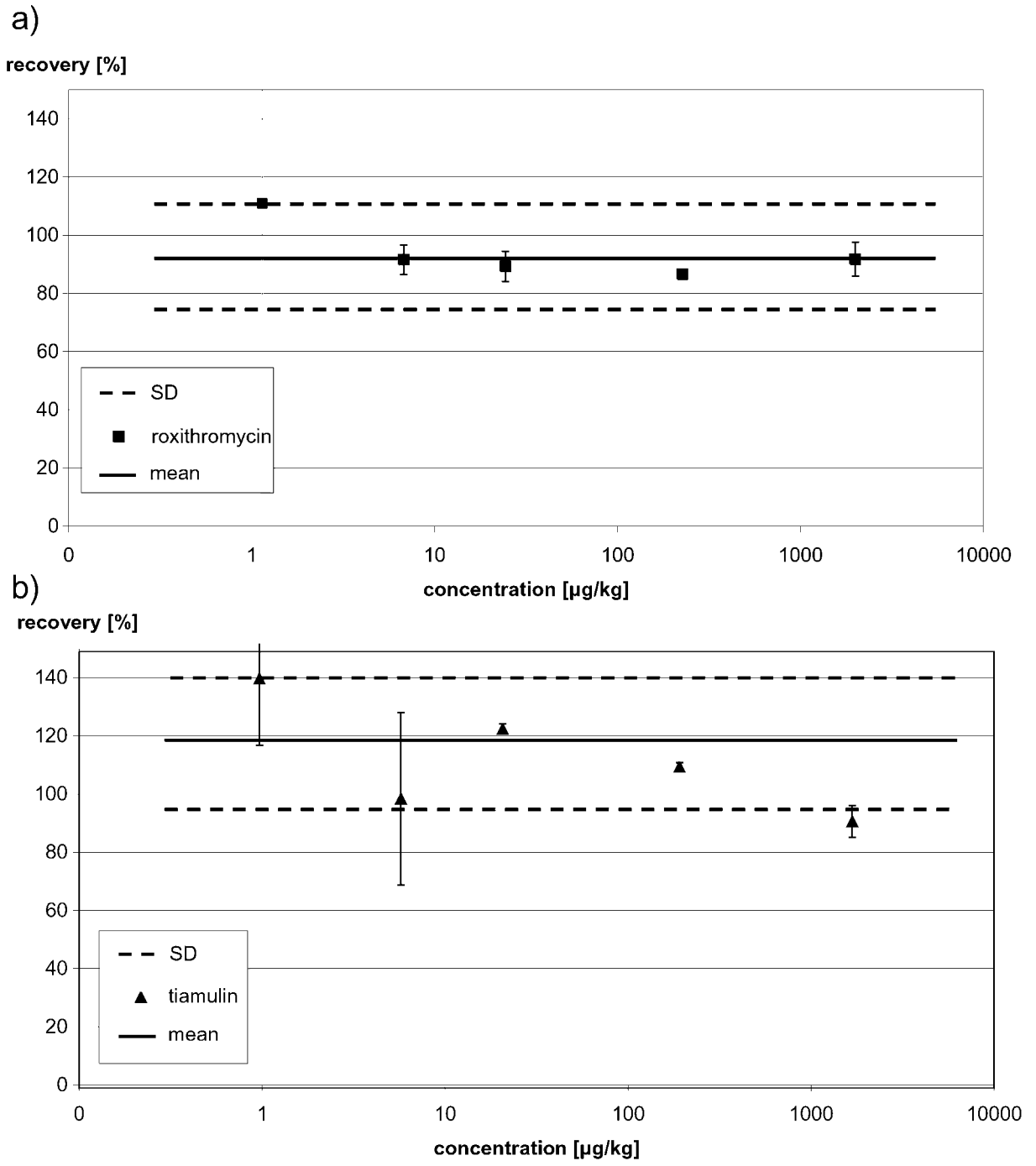

Fig. 2. Recovery for roxithromycin (a) and tiamulin (b) at five concentration levels (1, 6, 20, 200 and 2000 μg/kg soil). The standard deviation (SD) for three replicates is indicated by an error bar.

Table 1
SRM data and retention time of macrolides, ionophores and tiamulin

	RT (min)	Parent ion (amu)	Daughter ion (amu)	Collision energy (eV)
Oleandomycin	11.03	688.5	544.5	−20
Erythromycin	11.40	734.5	576.5	−22
Tylosin	12.13	917.1	772.5	−32
Roxithromycin	12.89	837.5	679.5	−25
Internal standard	13.10	763.5	605.5	−24
Tiamulin	13.52	494.6	192.3	−27
Monensin	22.97	688.5	461.3	−27
Ivermectin	23.80	892.5	569.5	−19
Salinomycin	24.05	768.7	733.6	−22

Table 2
Mean recovery, relative standard deviation (RSD) and limit of quantification (LOQ) (three extractions, repetitions for each concentration level) of macrolides, ionophores and tiamulin in soil

	Mean recovery (%)	RSD (%)	LOQ (μg/kg)
Erythromycin	43	23	1.4
Oleandomycin	38	51	1.4
Roxithromycin	94	19	1.0
Salinomycin	76	32	5.3
Tiamulin	118	18	0.6

Recoveries were determined at concentrations of 1, 6, 20, 200 and 2000 μg/kg manure. LOQ: S/N 10:1.

order of magnitude as the predicted environmental concentration (PEC_{soil}) of 0.3 μg/kg soil [25,26].

The PEC_{soil} was calculated with the following formula and assumptions:

$$PEC_{soil} = \frac{\rho_{manure} \cdot b_{antibiotic} \cdot Q_N}{\rho_{soil} \cdot 10\,000\,m^2 \cdot H_{depth}} \quad (1)$$

where density of manure (ρ_{manure}) is 1000 kg/m^3, concentration of antibiotics in the manure ($b_{antibiotic}$) is 43 μg/kg [21], area based load of manure (Q_N) is 20 m^3/ha, density of soil (ρ_{soil}) is 1800 kg/m^3 and penetration depth of the antibiotics in soil (H_{depth}) is 15 cm.

This is different to Kümmerer's own improved data of (PEC_{soil}) 3–180 μg/kg soil [27]. Further experiments, like time and temperature-dependent degradation experiments are necessary to obtain more information about the long-term stability of these compounds in soil.

4. Conclusions

A rugged and rapid multimethod with low LOQ has been developed to analyse macrolides, ionophores and tiamulin in soil. PLE extraction followed by a diol SPE clean-up step resulted in sufficient clean extracts, which were analysed by APCI LC–MS–MS. Recoveries for the macrolides ranged from 38 to 94%. For the ionophore salinomycin the recovery rate was 76±24%, while the pleuromutilin tiamulin had a recovery rate of 118±21%, i.e. its recovery is not significantly higher than 100%. Recoveries were not dependent on the concentration level. The limit of detection ranged from 0.2 to 1.6

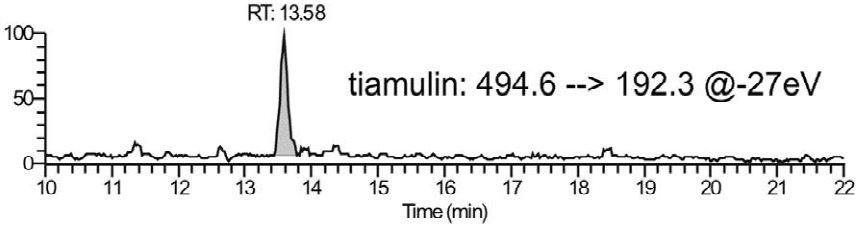

Fig. 4. APCI SRM trace of a soil sample which contains tiamulin (0.7 μg/kg). The soil was fertilised with manure, which contained tiamulin (43 μg/kg), 9 months before sampling.

μg/kg, and LOQs ranged from 0.6 to 5.3 μg/kg. In the tested samples tiamulin was found at concentrations of 0.7 μg/kg soil. The LOQs that were obtained in this study are lower than those published by Hamscher et al. [13] who investigated tetracycline antibiotics in manure and soil.

Acknowledgements

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